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DECREASED ESTROGEN HYDROXYLATION IN MALE RAT LIVER FOLLOWING CIMETIDINE TREATMENT

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Abstract—Administration of cimetidine (600 μmol/kg × 5) to adult male rats resulted in 55 and 25% decreases, respectively, in estradiol 2- and 16α-hydroxylation. The same treatment also decreased the activities of ethylmorphine demethylase, aryl hydrocarbon hydroxylase, aniline hydroxylase and heme oxygenase but did not inhibit the activities of 7-ethoxycoumarin de-ethylase and δ-aminolevulinic acid synthase or decrease cytochrome P-450 content. *In vitro* addition of cimetidine (10–300 μM) also inhibited estradiol hydroxylations, and the effect was additive in rats pretreated with cimetidine *in vivo*; the other enzymic activities studied were completely unaffected by *in vitro* addition of cimetidine. In contrast, there was no effect of cimetidine either *in vivo* or *in vitro* on any of these activities in female rats. The results point to a wide variation in the susceptibilities of different isozymes of cytochrome P-450 to inhibition by cimetidine and suggest that such differential susceptibilities are also highly dependent on the sex of the animal.

Cimetidine (*N*″-cyano-*N*-methyl-*N*′-[2[[(5-methyl-1*H*-imidazol-4-yl)methyl]thio]-ethyl]-guanadine) is a histamine H_2 receptor antagonist containing an imidazole ring. It is widely used in the treatment of duodenal and gastric ulceration, and in gastric acid hypersecretory conditions such as the Zollinger–Ellison Syndrome. Due to the widespread use of cimetidine for therapy of peptic ulcer disease, it is perhaps not surprising that some drug interactions between cimetidine and other compounds have been reported [1].

Inhibition of hepatic microsomal metabolism of many different substrates has been demonstrated following cimetidine administration to rats. Examples include the metabolism of 7-ethoxycoumarin [2, 3], antipyrine [4–6], hexobarbital and theophylline [7, 8], trimethadione [9], and aniline [10]. The hepatic metabolism of all these compounds is dependent, at least in part, on cytochrome P-450 isozymes of the microsomal mixed function monooxygenase system. Spectral studies have revealed that cimetidine binds to cytochrome P-450 in a fashion typical of "type II" ligands [2, 8, 11–13], although the total concentrations of cytochrome P-450 are generally unchanged [9, 10]. Furthermore, cimetidine itself, although predominantly excreted in the urine unchanged [14] or as cimetidine *N*-glucuronide [15], is also metabolized by cytochrome P-450 to *N*-desmethylcimetidine [16].

The majority of studies of cimetidine–cytochrome P-450 interactions have focused on the metabolism of exogenous compounds. Sporadic reports of gynecomastia [17], elevated prolactin levels [18], and decreased testosterone levels [19] have appeared following cimetidine treatment in men. Cortisol metabolism, as assessed by urinary excretion of 6β-hydroxycortisol, has also been demonstrated to be reduced modestly following cimetidine treatment [20, 21], but this finding was unconfirmed in another study [22]. Many of the hydroxylations involved in both the biosynthesis and catabolism of steroid hormones are catalyzed by various isozymes of cytochrome P-450 [23]. The interactions discussed above between cimetidine and cytochrome P-450 would suggest that at least some of the enzymatic steps in steroid metabolism may also be affected by cimetidine. Accordingly, we investigated the effect of cimetidine on the metabolism in rats of endogenous compounds, utilizing hepatic estrogen and androgen metabolism as model systems, and compared the results to the metabolism of exogenous compounds, cytochrome P-450 content, and heme pathway enzyme activities in the liver.

MATERIALS AND METHODS

Cimetidine base was a gift of Smith, Kline & Beckman (Philadelphia, PA, Lot No. 12-6-CFT-6-0). [2-^{3}H]E_2 (22.7 Ci/mmol) and [16α-^{3}H]E_1 (15–30 Ci/mmol) were obtained from the New England Nuclear Corp. (Boston, MA) and NADPH was from the Sigma Chemical Co (St. Louis, MO). Adult male or female Sprague–Dawley rats (200–250 g; mycoplasma-free) were purchased from Charles River (Wilmington, DE) and housed in the Rockefeller University Laboratory Animal Research Center at 23 ± 1° in 12-hr light cycled rooms (lights on at 7:00 a.m.) with free access to Purina rat chow and water. After acclimatization to these conditions for 1 week, animals were injected intraperitoneally with saline or various concentrations of cimetidine dissolved in saline. Rats were treated twice daily for 2 days (at 8:00 a.m. and 5:00 p.m.) and once on day 3. One hour later, animals were killed by decapitation;

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livers were perfused *in situ* with 30 ml of ice-cold saline and then excised, dried, weighed and homogenized to yield microsomal and mitochondrial fractions as described previously [24]. Microsomes were either used fresh for the determination of cytochrome P-450 content [25], δ-aminolevulinic acid (ALAS) activity [26] and heme oxygenase (HO) activity [27], or stored in liquid nitrogen prior to the determination of the activities of estrogen hydroxylases [28], aryl hydrocarbon hydroxylase (AHH; [29]), 7-ethoxycourmarin de-ethylase (7-EC; [30]), aniline hydroxylase (AHA;]31]) and ethylmorphine demethylase (EMD; [32, 33]). Spectral assays were performed on an Aminco Chance DW2A scanning spectrophotometer and fluorometric assays on a Hitachi MPFIV fluorescence spectrophotometer with an R928 photomultiplier tube. Protein content was determined by the method of Lowry *et al.* [34] using bovine serum albumin as a standard. Significance of difference between means was analyzed by Student's *t*-test, ANOVA and the Newman–Keuls test.

RESULTS

The effects of cimetidine treatment of male rats *in vivo* (600 μmol/kg × 5) and its addition *in vitro* (10–300 μM) on 2-hydroxylation and 16α-hydroxylation of estradiol are detailed in Table 1. *In vivo* treatment resulted in a 55% decrease in 2-hydroxylase activity when measured *in vitro*, but a smaller decrease (25%) in 16α-hydroxylation. *In vitro* addition of cimetidine to microsomal suspensions from saline-treated and cimetidine-treated rats resulted in reductions in both hydroxylation reactions, although the *in vitro* reductions seen in cimetidine-treated rats were proportionately less than those seen in saline-treated rats. In the case of 2-hydroxylation, *in vitro* treatment with 300 μM cimetidine resulted in the same degree of inhibition as rats treated *in vivo* with five doses of 600 μmol/kg.

The time course of the inhibition of estradiol 2-hydroxylase following *in vivo* administration of cimetidine is illustrated in Fig. 1. The modest inhibition of 16α-hydroxylation caused by *in vivo* treatment with cimetidine at various concentrations of substrate is shown in Fig. 2. Note the level of activity in control microsomes which is consistent with the low activity of 16α-hydroxylation in males. In contrast, the activity of 2-hydroxylation rose with increasing substrate concentrations in microsomes from saline-treated rats and was inhibited in microsomes from cimetidine-treated rats (Fig. 2). Transformation of the data from Fig. 2 yielded the Lineweaver–Burk plots for the inhibition of 2-hydroxylation (Fig. 3A) and 16α-hydroxylation (Fig. 3B) of estradiol. In both cases, the apparent K_m was unchanged, with a modest increase in V_{max} for 2-hydroxylation but not for 16α-hydroxylation following cimetidine treatment.

Heme pathway enzymes (ALAS and HO), cytochrome P-450 concentrations and four drug-metabolizing enzyme activities were determined in livers of male rats treated with saline or various concentrations of cimetidine. The results are shown in Table 2. Cytochrome P-450 concentrations were not affected by low doses of cimetidine, although they were minimally elevated at the highest dose (600 μmol/kg × 5). HO activity declined modestly but progressively with increasing cimetidine concentrations, but ALAS activity was not altered consistently. EMD and AHH activities were reduced about 50% by all doses of cimetidine, whereas the inhibition of AHA was pronounced and dose-responsive; 7-EC activity was completely unaffected.

Table 1. Effect of cimetidine on liver microsomal estradiol hydroxylases from normal and cimetidine-treated male rats

Treatment	Cimetidine concentration (μM)	Formation of ^3H_2O (%)	
		[2-^{3}H]E_2	[16α-^{3}H]E_2
Control	—	46.2 (47.4)	7.6 (16.4)
	10	45.7 (47.0)	7.3 (18.3)
	30	42.7 (44.1)	5.4 (17.7)
	100	33.7 (35.3)	5.7 (14.4)
	300	21.6 (23.9)	3.4 (12.0)
Cimetidine	—	21.2 (23.9)	5.7 (16.9)
	10	17.8 (20.5)	4.3 (14.3)
	30	16.2 (18.7)	3.0 (13.2)
	100	13.4 (15.7)	4.5 (13.0)
	300	9.6 (12.1)	2.9 (9.7)

The ^{3}H-labeled steroids were incubated for 30 min at 37° with NADPH (0.16 mM) and liver microsomes (0.5 mg protein) from control or cimetidine (600 μmol/kg)-treated male rats, and ^3H_2O formation was determined as described in Materials and Methods. Results are the averages of two experiments (range <10% of averages). Values in parentheses are percentages of ^{3}H-radioactivity that could not be extracted from the medium by ether.

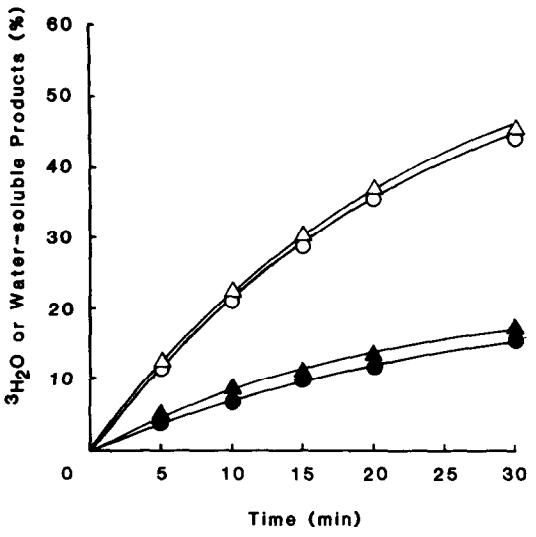

Fig. 1. Time course of the effect of cimetidine on the rate of metabolism of estradiol in male rat liver microsomes. Microsomes (0.5 mg) from the livers of rats treated with saline (open symbols) or cimetidine (600 μmol/kg × 5; closed symbols) were incubated with [2-^{3}H]E_2 (9.2 μM) and NADPH (0.16 mM) for various time periods, and the percentage converted to water-soluble products (△, ▲) or ^3H_2O (○, ●) was determined. Results are the averages of two experiments (range <10% of averages). Other conditions are described in the text.

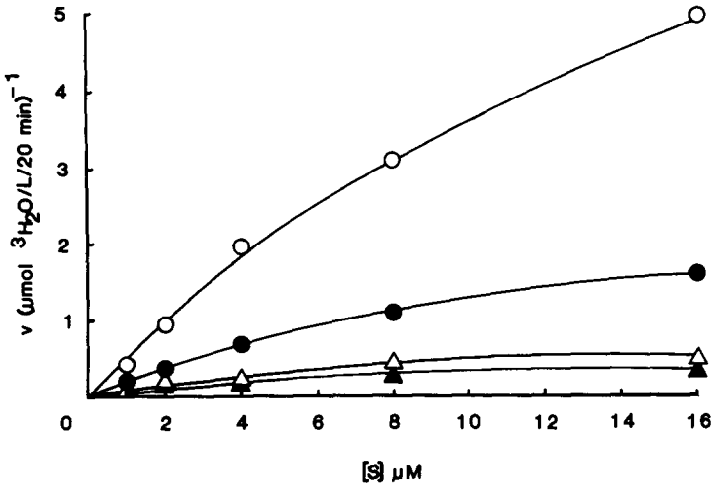

Fig. 2. Effect of treatment with cimetidine on estradiol 2- and 16α-hydroxylase in male rat liver microsomes. Microsomes from rats treated with saline (open symbols) or cimetidine (600 μmol/kg × 5; closed symbols) were incubated for 20 min with various concentrations of [2-^{3}H]E_2 (○, ●) or [16α-^{3}H]E_2 (△, ▲) under the conditions described in the legend of Fig. 1, and the rate of ^3H_2O formed was measured as described in Materials and Methods. Estradiol hydroxylase activity is expressed as μmol ^3H_2O formed/L/20 min and is the average of two experiments (range <10% of average).

In vitro addition of cimetidine (10–300 μM) to microsomes from saline-treated rats resulted in no significant inhibition of the activities of the four drug-metabolizing enzymes (data not shown). In no case was the inhibition during *in vitro* treatment with cimetidine additive when tested on microsomes from rats treated *in vivo* with cimetidine; in the case of EMD and AHA activities, *in vitro* cimetidine was less inhibitory than *in vivo* treatment, whereas the inhibition of AHH activity was identical and 7-EC activity was completely unaffected (data not shown).

In contrast to the results with male rats, there was no effect after *in vivo* treatment of female rats with cimetidine (600 μmol/kg × 5) on 2-hydroxylation of estradiol by hepatic microsomes when compared to microsomes from saline-treated control rats (Fig. 4). Similarly, there was no significant effect by *in vivo* cimetidine treatment on 16α-hydroxylation of estradiol in female rats (water-soluble products: 12.5% for control and 11% for cimetidine-treated: ^3H_2O formation: 6% for control and 4% for cimetidine-treated). The activities of the heme pathway enzymes (ALAS and HO) and of four drug-metabolizing enzymes as well as the cytochrome P-450 concentrations in hepatic microsomes from female rats are presented in Table 3. Basal concentrations of cytochrome P-450 were approximately 50% of those observed in male rats, whereas the activities

Table 2. Effect of *in vivo* cimetidine treatment on heme pathway and drug-metabolizing enzymes in male rat liver

Enzyme concentration or activity	Cimetidine (μmol/kg)			
	0	200	400	600
Cytochrome P-450 (nmol/mg protein)	0.79 ± 0.04	0.84 ± 0.08	0.81 ± 0.04	1.03 ± 0.05*
Heme oxygenase (nmol bilirubin/mg protein/hr)	6.49 ± 0.35	5.71 ± 0.32	4.75 ± 0.23*	4.16 ± 0.05*
δ-Aminolevulinic acid synthase (μmol ALA/90 min/mg protein)	0.39 ± 0.03	0.56 ± 0.06*	0.30 ± 0.02	0.25 ± 0.01*
Ethylmorphine demethylase (μmol formaldehyde/mg protein/hr)	0.50 ± 0.04	0.25 ± 0.05*	0.23 ± 0.06*	0.26 ± 0.04*
Aniline hydroxylase (nmol *p*-aminophenol/mg protein/hr)	126 ± 23.95	85 ± 12.87*	59 ± 5.45*	22 ± 8.34*
Aryl hydrocarbon hydroxylase (nmol 8OH-benzopyrene/mg protein/hr)	0.32 ± 0.05	0.12 ± 0.02*	0.20 ± 0.03*	0.22 ± 0.03*
7-Ethoxycoumarin de-ethylase (μmol/mg protein/hr)	4.11 ± 0.50	5.80 ± 0.65	4.25 ± 0.64	3.79 ± 0.47

Male rats were treated i.p. with five doses of saline or the indicated concentrations of cimetidine. Mitochondrial and microsomal fractions were prepared and the activities of the indicated enzymes were determined as described in Materials and Methods. Means ± SEM of three animals per treatment group are presented.

* $P < 0.05$, versus respective controls (Newman–Keuls test).

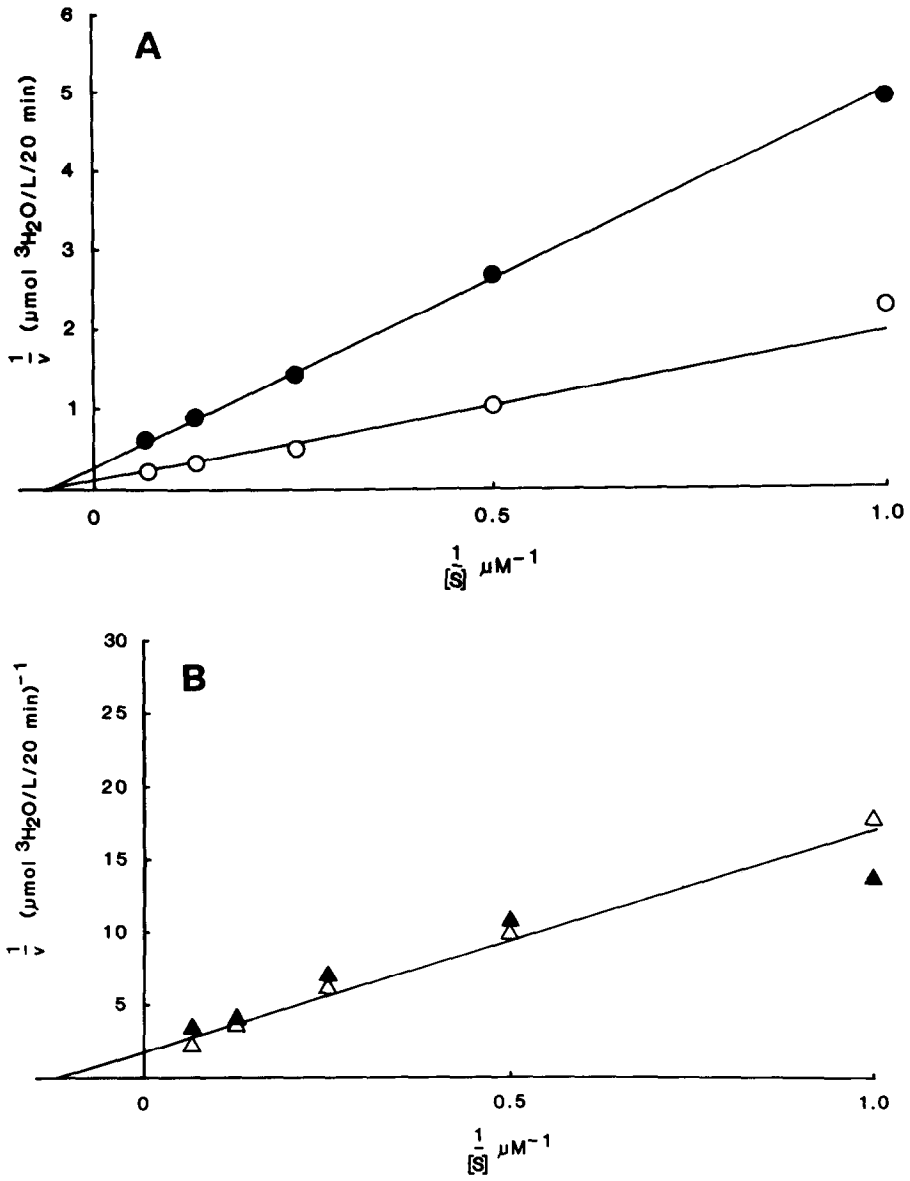

Fig. 3. Lineweaver–Burk plots for the inhibition of hepatic estradiol 2-hydroxylase (A) and 16α-hydroxylase (B) after treatment of male rats by cimetidine. Experimental conditions and symbols are identical to those in Fig. 2. Results are the averages of two experiments (range <10% of averages).

of HO and ALAS were comparable (cf. Table 2). Compared to male rats, the basal hepatic microsomal activities of EMD, AHA, AHH and 7-EC in the females were reduced by 80, 60, 90 and 70% respectively. However, none of these activities were affected by *in vivo* (200–600 μmol/kg × 5) or *in vitro* (10–300 μM) cimetidine treatment (data not shown).

DISCUSSION

We have demonstrated that *in vivo* administration of cimetidine (600 μmol/kg × 5) to male rats resulted in marked inhibition of 2- and modest inhibition of 16α-hydroxylation of estradiol, and alterations in the activities of HO, EMD, AHH and AHA in hepatic microsomes; cytochrome P-450 concentrations and ALAS and 7-EC activities were unaffected. *In vitro* addition of cimetidine to microsomes from male rat livers also inhibited estradiol hydroxylations, and the effect was additive when tested on microsomes from rats pretreated with cimetidine *in vivo*. In contrast to estrogen metabolism, the activities of the four drug-metabolizing enzymes were unaffected by *in vitro* addition of cimetidine, and no additive effect was observed when tested on microsomes from rats pretreated with cimetidine *in vivo*.

These results confirm the findings of other investigators that *in vivo* administration of cimetidine to male rats results in inhibition of various cytochrome P-450-dependent drug metabolizing activities

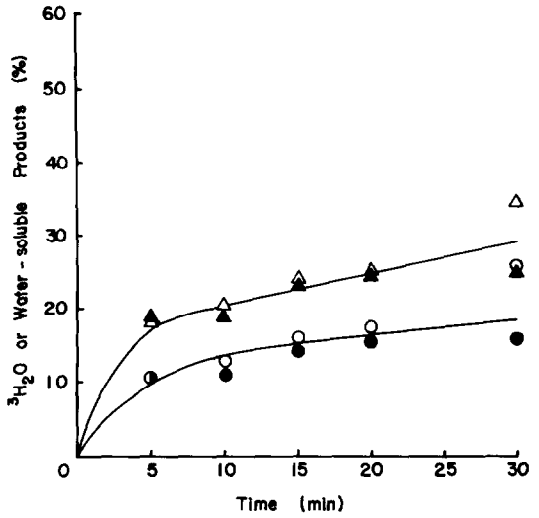

Fig. 4. Effect of treatment of female rats with cimetidine on the rate of metabolism of estradiol by liver microsomes. Experimental conditions are identical to those of Fig. 1 except that the microsomes were prepared from female rats treated with saline (open symbols) or cimetidine (600 μmol/kg × 5; closed symbols). Data points are the percentage of [2-^{3}H]E_2 converted to water soluble products (△, ▲) or ^3H_2O (○, ●) from two separate experiments (range <10% of average).

[2, 3, 9, 10] and extends this concept to include the metabolism of the endogenous steroid, estradiol. As noted in other reports, the content of cytochrome P-450 [9, 10, 35] and the activity of ALAS [10] were not decreased following *in vivo* cimetidine treatment. HO activity was decreased significantly ($P < 0.05$) with cimetidine doses of 400 and 600 μmol/kg, but, in agreement with previously published data [10], was unaffected by doses of 200 μmol/kg; both these results are in contrast to the results of Reichen *et al.* [35] who reported induction of HO activity following higher doses of cimetidine (900 μmol/kg b.i.d. for 3 days).

The lack of inhibition of drug-metabolizing enzyme activity following *in vitro* addition of cimetidine is in marked contrast to other reports [2, 3, 8, 11–13]. However, the majority of such studies have utilized cimetidine concentrations in the millimolar ranges, whereas plasma concentrations following *in vivo* administration of cimetidine are generally 10–1000 times lower [36, 37]. Despite the absence of any effect of these doses on drug-metabolizing activities, it is noteworthy that they were sufficient to substantially inhibit the 2-hydroxylation of estradiol in male rat liver, suggesting that the isozyme of cytochrome P-450 responsible for this activity in the rat may be unusually sensitive to cimetidine. Although we have only determined enzyme activities in this manuscript, the demonstration of a ligand type II interaction between cimetidine and cytochrome P-450 (2, 8, 11–13) favors a direct inhibitory action of cimetidine rather than a change in protein concentration.

In contrast to the diverse effects of cimetidine observed in male rats, there was no effect in female rats on any activity studied after either *in vivo* or *in vitro* treatment with cimetidine. There was, however, a generalized decrease in cytochrome P-450 content and drug-metabolizing enzyme activities. It is known that the pattern of hepatic cytochrome P-450 isozyme activities is sex dependent in the rat and that the pattern of growth hormone release is an important determinant in the genesis of such sex dependence [28, 38, 39]. Sex-specific cytochrome P-450 isozymes can also be induced by steroid hormones [40, 41].

We interpret these results to suggest that cimetidine may exhibit multiple differential inhibitory actions on the metabolism of various endogenous and exogenous substrates in the rat. The resulting inhibitions are dependent on dosage, method of administration and the sex of the animal, and likely reflect differing susceptibilities of different isozymes of cytochrome P-450 to the action of cimetidine, as has been reported previously [42]. Future studies to determine whether these changes in estrogen metabolism also occur in human subjects following treatment with cimetidine are in progress.

Table 3. Effect of *in vivo* treatment with cimetidine on heme pathway and drug-metabolizing enzymes in female rat liver

Enzyme concentration or activity	Cimetidine (μmol/kg)			
	0	200	400	600
Cytochrome P-450 (nmol/mg protein)	0.42 ± 0.08	0.50 ± 0.12	0.57 ± 0.04	0.44 ± 0.03
Heme oxygenase (nmol bilirubin/mg protein/hr)	4.24 ± 0.51	5.64 ± 1.06	4.74 ± 0.95	4.68 ± 1.43
α-Aminolevulinic acid synthase (μmol ALA/90 min/mg protein)	0.54 ± 0.07	0.58 ± 0.03	0.51 ± 0.01	0.56 ± 0.05
Ethylmorphine demethylase (μmol formaldehyde/mg protein/hr)	0.11 ± 0.02	0.17 ± 0.04	0.15 ± 0.02	0.15 ± 0.04
Aniline hydroxylase (nmol *p*-aminophenol/mg protein/hr)	46.9 ± 4.36	55.3 ± 3.30	52.61 ± 3.57	48.40 ± 3.98
Aryl hydrocarbon hydroxylase (nmol 8OH-benzopyrene/mg protein/hr)	0.03 ± 0.005	0.05 ± 0.008	0.07 ± 0.012	0.04 ± 0.01
7-Ethoxycoumarin de-ethylase (μmol/mg protein/hr)	1.31 ± 0.05	1.45 ± 0.13	1.50 ± 0.11	1.36 ± 0.09

See legend of Table 2 for experimental conditions. Means ± SEM of three animals per treatment group are presented.

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